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THE EFFECT OF URETHAN ON HYDRA

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Although urethan (ethyl carbamate) is a common invertebrate anesthetic, very little is known about its mechanism of action. It is frequently used as a reversible anesthetic; that is, urethan may be applied to the animal which will be seen to relax; an experimental maneuver is performed; and the animal recovers and shows no long-term ill-effects after the urethan is washed away. However, in preliminary, but unreported, experiments with hydra (prior to Josephson and Macklin, 1969; and Macklin and Josephson, 1971), urethan was used to relax animals so as to facilitate experimental manipulation. It was discovered that the transepithelial potential of animals in urethan reversed; that is, the inside or gut of the animal is normally positive relative to the surrounding medium, but urethan would cause the inside to become negative with respect to the outside medium. Because of this, none of our previous experiments were conducted using urethan, and these preliminary results were ignored because of the difficulty they presented to the main experimental line. However, the effect of urethan is still of interest, not only because urethan is a common experimental drug but also because of this curious effect.

The literature is rather uniform in declaring that urethan acting on nervous tissue has *no* effect on transmembrane potentials and only has an effect on the conduction of action potentials, thus acting as an anesthetic (*e.g.,* Barker and Gainer, 1973; Il'inskii and Tertyshnik, 1960; Iwase, 1957; Kobayashi, 1963; Rudolph, 1953; Salmoiraghi and Weight, 1967; Sorokina, 1958; Thesleff, 1956). The same authors go on to say that the effect of urethan is a postsynaptic junctional effect. There is also a report by Wilbrandt and Schatzmann (1960) that whereas low electrolyte concentration media cause the cation permeability of red blood cells to increase, urethan partially blocks this effect.

The literature references quoted above typically used urethan in concentrations between 1 and 3% w/v (112 to 337 mM), which are similar to the concentrations used with coelenterates. Therefore, there seems to be a difference in the effects on nervous tissue and the effect on hydra epithelia. One clue to the possible consistency of effects is that urethan is claimed to stabilize the DC-potential across nerve membranes by an effect on sodium conductance; and sodium transport is felt to be responsible for the transepithelial potential reported in hydra (Macklin, 1967; Macklin and Josephson, 1971).

The experiments to be described were planned to gain additional insight into the mechanism of action of urethan on hydra.

Materials and Methods

Hydra were cultured in the manner of Loomis and Lenhoff (1956). Experimental animals were fed daily with newly hatched artemia. All experiments in

TABLE I

Experimental solutions used. All concentrations are expressed in mM/L.

Solution name	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	$SO_4^=$	HCO_3^-	$EDTA^=$	Urethan
Culture	1.44		1.5		3.0		1.2	0.12	
Normal	1.5		1.5		3.0		1.5		
M	1.0	0.1	1.0	0.1	2.3		1.0		
Haynes	12.0	0.6	12.6	1.8	37.2	1.8	0.6		
Low calcium	1.44		0.15		0.3		1.2	0.12	
Urethan (2% w/v)	1.5		1.5		3.0		1.5		224

this series were conducted at 20 to 24° C with *Hydra oligactus* starved for 24 hours prior to use.

A number of solutions varying in ionic composition were used in the study (Table I). "Normal solution" which is the standard for comparison (Macklin and Josephson, 1971) differs from the culture solution primarily in the absence of EDTA. EDTA is included in the culture medium to remove heavy metal ions which can be toxic in small concentrations (Loomis and Lenhoff, 1956). All solutions were prepared in distilled water with reagent grade chemicals.

For the preparation of epidermal or gastrodermal isolates, a modification of an unpublished technique developed by M. Cyrlin and A. L. Burnett (Northwestern University, Evanston, Illinois) was used. The separation technique was checked by looking at whole mount preparations for the presence of gastrodermal pigment in epidermal pieces, and by looking for the characteristic mucous droplets of epithelial cells in gastrodermal pieces. Histological preparations confirmed the validity of the technique. Pieces which showed poor homogeneity of tissue on inspection were not used in experiments. In addition, for electrophysiological studies on regenerates, it was necessary that the piece form a cavity. In most cases, by 24 hours there was an identifiable cavity in the epidermal and gastrodermal isolates. The cell layer separation technique is summarized below.

To obtain gastrodermal explants, intact animals are placed in M solution (Muscatine, 1961) adjusted to pH 2.5 with 0.1 N HCl. After one minute in the acid M solution, the animals are transferred to Haynes solution (Haynes and Burnett, 1963). The epidermis contracts and may be removed by a combination of gentle swirling and manipulation with glass needles. Gastrodermal explants are then placed in fresh Haynes solution for 24 hours prior to use.

To obtain ectodermal explants, intact animals are placed in 10^{-5} M reduced glutathione in culture solution until a yawning response is obtained. Animals are everted by grasping the basal disc with number 5 jewelers forceps and pulling it through the mouth. The animals are transected at the subhypostomal level and placed in the low calcium solution. Agitation in this solution causes the gastrodermal layer to separate from the ectodermal layer. Ectodermal explants are placed in culture solution for 24 hours prior to use. Ectodermal explants all return to their normal orientation prior to the formation of a regenerate; *i.e.*, the usual outside of the regenerate faces the external medium. This mechanism of re-turning was previously reported by Macklin (1968).

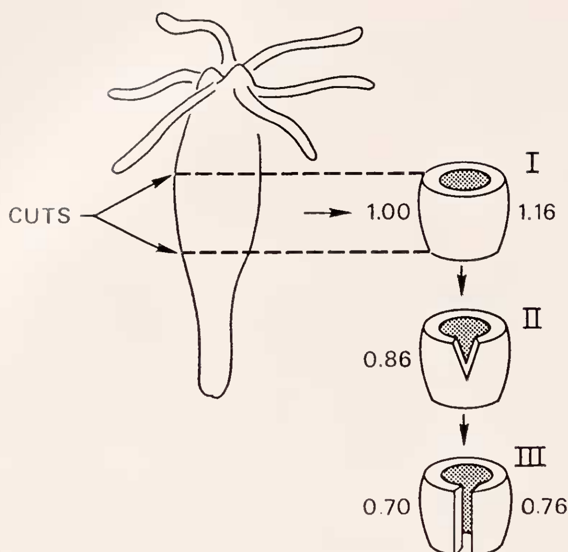

FIGURE 1. Method of preparing *Hydra* regenerates. The animal was cut twice as shown in the first diagram of the sequence and the mid-section of the animal (gastric region) was used. For the experiment in which the animal was cut longitudinally, configurations I, II and III identify the method of cutting. Configuration I is the regenerate as removed from the animal; in configuration II a longitudinal cut half-way down the regenerate was made; and in configuration III the longitudinal cut was completed. The numbers shown adjacent to the configurations give the relative length of the animal normalized to the length of configuration I in normal solution. The lengths on the left side are normalized lengths for maximal relaxation and those on the right side of the figure for maximal lengths in 2% w/v urethan. Data is from Table II.

To measure electrical potentials across the body wall, glass micropipettes filled with 3 M KCl with chlorided silver wire electrodes were used. Potentials were measured with the electrode tip in the gut cavity of the animal or regenerate using a high input impedance DC operational amplifier and displayed either on an oscilloscope or curvilinear chart recorder. Potentials were also read from a digital voltmeter. To verify representative resting potentials for each of the various conditions studied, preliminary testing established the time required for the potential to become stable. For a five minute test period, the resting potential was measured at 3, 4, and 5 minutes after a solution change, the average of these three values was used as a measure of the resting potential. Similarly, for a 20 minute test period, the resting potential was recorded 17, 18, 19, and 20 minutes after a solution change. All data reported are the result of the analysis of data from multiple animals for each separate experiment. Solution changes were such that we were assured of a greater than 99.9% exchange of solution. The experimental chamber used is the same one previously used in experiments reported in Macklin and Josephson (1971).

When the length of animal pieces were being measured, the pieces were examined immediately after preparation (Fig. 1) under a dissecting microscope with

a micrometer eye piece. Each piece was watched and its length measured at its maximum value as the animal contracted and relaxed. The diagram in Figure 1 illustrates the various cuts made.

Results

Relaxed length of hydra segments

Hydra have the usual arrangement of myotomes or muscle fibers as in other coelenterates; that is, an inner circular or helical layer and an outer longitudinal layer. This is the common pattern found in vertebrate tubular structures as well. Two effects are of interest here. The first is the relationship between the continuity of the inner helical layer of muscle fibers and the relaxed length of the animal; and the second is the effect of urethan on animal length. It is generally assumed that urethan is strictly an anesthetic and causes passive relaxation of the animal. It was this hypothesis that was being examined.

Figure 1 shows the sequence of cuts that were made in this series of experiments. An animal had its hypostome and peduncle surgically removed and was then immediately examined under a dissecting microscope. Lengths were measured as follows. In the first series of experiments the length of the excised segment (Fig. 1, configuration I) was observed and the maximum relaxed length was recorded. The segment was cut half-way down the length of the cylinder (II) and once again the length of the maximum relaxed length of the segment was recorded. The cut was then extended all the way down (III), the animal was continually observed, and its maximum length recorded. This final segment (configuration III) was then placed in 2% w/v urethan and its maximum length was once again recorded. The summarized results are recorded as Experiment 1 in Table II. In a second series, configuration I segments were not cut longitudinally but had their maximum lengths measured in the normal solution and in the urethan solution (Experiment 2, Table II).

Normalized results shown in Figure 1 summarize the data from 34 separate animals with their lengths all normalized to the length of the animal in its maxi-

Table II

The mean length of fully relaxed Hydra oligactus *pieces is contrasted for intact gastric segments (configuration I) and the same segments, cut as shown in Figure 1, in culture medium and in culture medium with 2% w/v urethan added. The values of* P *shown are for Student's* t*-test for paired data for a comparison of each length with the immediately preceeding length in the table.*

	Configuration	Urethan	Length ± s.e.	*P*
Experiment 1 (n = 22)	I	No	2.5 ± 0.2 mm	
	II	No	2.1 ± 0.1 mm	<0.001
	III	No	1.6 ± 0.1 mm	<0.001
	III	Yes	1.9 ± 0.1 mm	<0.01
Experiment 2 (n = 12)	I	No	2.6 ± 0.2 mm	
	I	Yes	3.0 ± 0.2 mm	<0.001

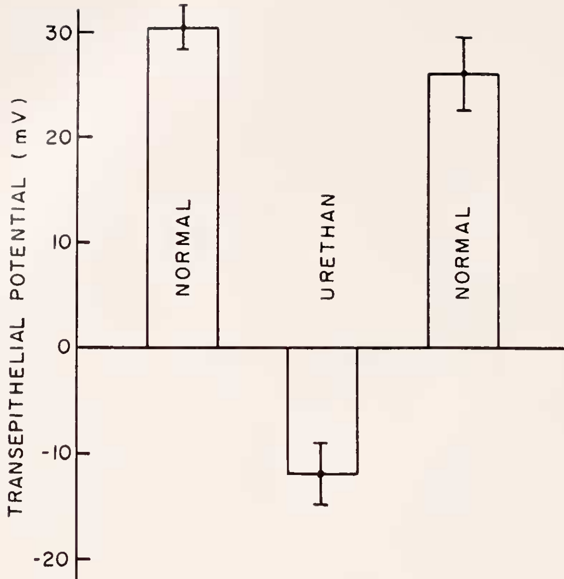

FIGURE 2. Effect of 2% w/v urethan on transepithelial potential in *Hydra oligactis*. Hydra were held on the special glass holder and potentials measured as described in Josephson and Macklin (1971). Normal solution was placed on the outside of the animal, exchanged for the 2% w/v urethan and then exchanged for normal in sequence. The mean ± standard error for seven animals are shown. Using Student's *t*-test, the difference between the transepithelial potential in normal and urethan solutions is significantly different ($P < 0.001$). The two values in normal solution are not significantly different ($P > 0.2$). Animals were in each test solution for twenty minutes and readings were taken every minute for the last four minutes. The method is described in the text.

mum relaxed length in configuration I. Note that the maximum length decreases as continuity of the circular muscle layer is destroyed. The length of the longitudinally cut animal is only 70% of the maximum length that the animal can achieve without this cut. In addition, once the animal is placed in urethan, whether it is a segment with the circular muscles intact (I), or if the continuity of the circular muscles are completely destroyed (III), there is an increase in length in both cases. For the intact circular pattern, there is a 16% increase due to urethan, and for the severed pattern it is a 9% increase.

Two points emerge from these observations. First, complete relaxation or elongation of hydra is the result of at least two events: the relaxation of the longitudinal muscle layer, and the active contraction of the inner circular muscles. The active contraction is apparent because once the continuity of the inner muscle layer is severed, the maximum relaxed length of the animal is reduced (Experiment 1, Table II). The results suggest that 70% of the lengthening of the relaxed animal is due to the relaxation of the outer layer and an additional 30% lengthening is due to a contraction of the inner muscle layer. Secondly urethan is not solely an outer longitudinal muscle layer anesthetic as generally assumed. That

is, it not only has a relaxing effect, but it appears to have an active effect causing the inner muscle layer to contract. Alternatively, we could assume that the outer longitudinal muscles do not fully relax unless they are placed in urethan. However, the fact that severing the circular muscles causes a statistically significant reduction in the lengthening ability of the animal points to the conclusion that the circular muscles are partly responsible for the length of the fully relaxed animal.

Effect of urethan on transepithelial potentials

These results are the most interesting and least explicable in this series of experiments. Without exception, in all of the experimental manipulations, urethan causes the normal positive transepithelial potential in hydra to become negative (Fig. 2). Also, urethan causes the contraction pulses (CP's) to diminish in size (Fig. 3). In other recordings, occasional biphasic and inverted contraction pulses were seen. The presence of a biphasic CP is uncommon in our experience.

Since several different manipulations were performed, they will be discussed in turn. The effect of urethan on intact hydra was determined using the special glass holder previously described by Josephson and Macklin (1969); and the effect of urethan on gastric region regenerates and epidermal and gastrodermal cell layer isolates was determined using micropipette impalement into the gut cavity. In all experimental situations, the same experimental protocol was followed. The animal was first placed in normal solution for a time period sufficient for the transepithelial resting position to stabilize; the bathing solution was changed to the urethan solution, the animal was held in that solution for 5 to 20 minutes, and then the animal was returned to the normal solution. The potential in the urethan solution differed significantly from the potential in the normal solution—both the initial and the final normal solution changes—for all animal forms tested except for gastrodermal layer isolates.

When the transepithelial resting potential was measured for intact hydra (Figs. 2 and 3) or for regenerates (Fig. 4), the same result was observed. Urethan

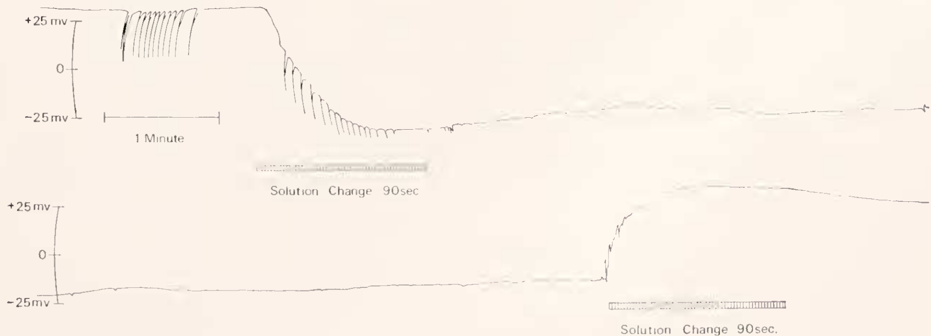

FIGURE 3. Tracing of recording from a curvilinear recorder showing the transition from normal solution to urethan and back to normal solution. The original recording was traced for clarity. Note in particular how the contraction pulses (CP's) decline in the urethan solution and the rapidity of the change in potential when solutions are changed.

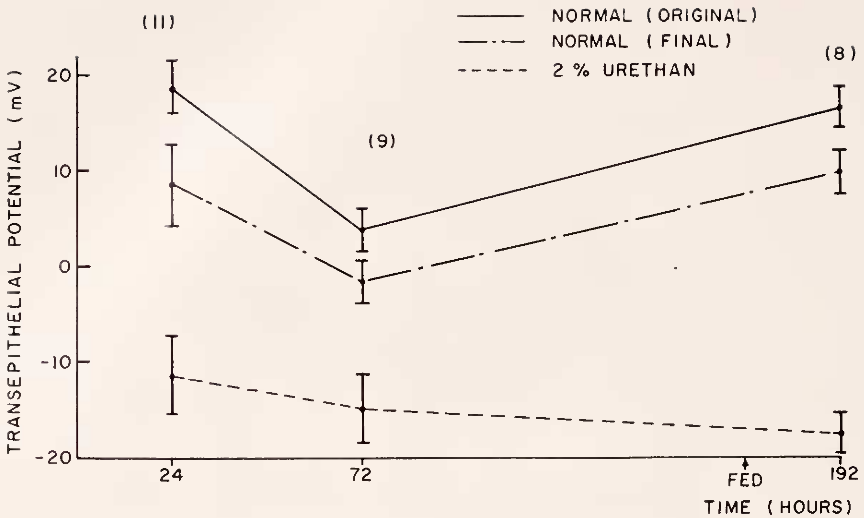

FIGURE 4. Effect of urethan on transepithelial potential in *Hydra* regenerates. For this experiment glass micropipettes were used to measure the transepithelial potential in hydra regenerates. For the sequence shown, eleven animals were used; however, three animals were lost so that the value for N differs during the sequence. From this data, it can be seen that the transepithelial potential is significantly less in the urethan solution than in the normal solution for each test time ($P < 0.001$). Also, it is interesting to note that there is initially a decrease in transepithelial potential; and following feeding the potentials increase. The experiment was conducted by first placing the animal in normal solution, then in 2% w/v urethan, and then back into the normal solution. Animals were held in each solution change for five minutes and the values used for determining the potential were recorded every minute for the last three minutes using the method described in the text.

causes the resting potential to drop significantly ($P < 0.001$) and to reverse polarity. The gut potential which is normally positive with respect to the medium becomes negative. CP's also always diminish in size and may become inverted or biphasic. The result does not appear to be analogous to previously reported effects of osmotic pressure (Macklin and Josephson, 1971). This is based on the previous observation that an osmotic pressure of 67.5 mOsmol or greater produced irreversible short term changes in transepithelial resting potential, whereas urethan does not ($P > 0.2$). It is therefore hypothesized that increasing osmotic pressure in the bathing medium with sucrose, a nonpermeate solute, has an entirely different effect on the ion transporting system from increasing the osmotic pressure with urethan, a highly permeate solute.

To localize the effect of urethan in the hydra body wall, a second series of experiments were conducted in which cell layer isolates were compared with regenerates and intact animals (Table III). All four animal configurations were tested under the same conditions using micropipette electrodes inserted through the body wall into the gut cavity. Except for the gastrodermal layer isolate, there is a significant ($P < 0.001$) reduction in transepithelial resting potential attributable to the urethan. Urethan does not effect the transepithelial potential of gastrodermal

cell layer isolates. Only cell layer isolates with an easily identifiable cavity 24 hours after preparation were used in these experiments.

Discussion

All the results point to the conclusion that there are at least two effects of urethan on hydra. The effect on electrical activity can be further subdivided into a change in transepithelial potential and an effect on contraction pulses. The second effect is on the contraction of the gastrodermal muscle layer.

Previous results (Macklin and Josephson, 1969) demonstrate that the normal resting potential in hydra is related to a flow of sodium ions from the culture medium to the gut—the sodium being transported by an electrogenic mechanism. It has been further hypothesized (Macklin, 1967) that the gastrodermal cell layer is also capable of actively transporting sodium in the opposite direction; but there is an adaptational effect of the cell layers based on their location in the animal such that in normal free-living hydra the net flow of sodium is inward. This is clearly the required direction for net ion transport for osmotic equilibrium. However, urethan causes the potential difference to reverse; that is, the outside becomes negative relative to the inside of the animal. This further supports the existence of two transport systems. The usual net flow of sodium ions is from the medium into the animal tissue, therefore it is possible that urethan abolishes the flow of sodium from the medium to the epithelial cell layer. What is observed in the presence of urethan is only the sodium current from the gut cavity into the animal tissue. Since animal tissue and the gut have a higher sodium concentration than the medium (Steinbach, 1963), simply abolishing active inward flow would result in a reversal of the transepithelial potential because the Nernst potential would

Table III

Effect of urethan on mean ± standard error of transepithelial potential in Hydra oligactus. *For this comparison all animals were impaled with glass micropipettes and sequentially placed in normal, urethan, and normal solutions. Recording techniques are described in the text. The transepithelial potentials in urethan statistically differ from the initial and final potentials in the normal solution for the first three experiments (Student's* t*-test,* $P < 0.001$*). However, when only the gastrodermal layer is present, urethan does not effect the transepithelial potential* ($P > 0.2$ *for the first two values and* $P > 0.8$ *for the second two values). Animals were in each solution change for five minutes; measurements were recorded each minute for the last three minutes.*

Animal configuration	Solution		
	Normal (initial)	2% Urethan	Normal (final)
Intact animal (n = 11)	20.8 ± 1.1 mV	−8.0 ± 1.6 mV	16.3 ± 2.0 mV
24 hour regenerate (n = 11)	18.8 ± 2.7	−11.3 ± 4.0	8.5 ± 4.2
24 hour epidermal layer isolate (n = 10)	11.2 ± 2.2	−3.5 ± 3.1	12.9 ± 3.1
24 hour gastrodermal layer isolate (n = 10)	1.0 ± 0.6	0.1 ± 0.5	0.1 ± 0.4

remain. Also, when isolated cell layers were used (Table III), it was noted that for the isolated epithelial layer the potential reversed, whereas for the gastrodermal cell layer there was essentially no effect on transepithelial potential. This implies that even if there is a bidirectional sodium current in the intact animal with the gastrodermal cell layer pumping in opposition to the epidermal cell layer, when the gastrodermal layer is removed there should only be a unidirectional sodium current in the isolated epithelial cell layer. Therefore, a more consistent conclusion based on the data is that urethan causes the epithelial cell layer to become leaky so that there is then a net flow of sodium ions from the tissue into the external medium. The negative transepithelial potential then is a measure of this passive flow. This is consistent with the observation that when the animal is transferred from urethan solution back to normal solution the final potential is slightly, but not significantly less than the initial potential. This is similar to previous results (Macklin and Josephson, 1971) with animals transferred from solutions containing calcium to solutions without calcium, where there was apparently structural damage due to the short term absence of calcium in the medium. An alternative explanation for the transepithelial potential reversal in the presence of urethan is that urethan inhibits the electrogenic transport system. The normal diffusion of sodium out of the epithelial cells due to the electrochemical gradient would then account for the apparent damage after removal of urethan from the bathing medium.

The effect of urethan on the contraction pulses is likewise of interest. This effect has been previously noted in other experimental preparations (Macklin and Josephson, 1971) where biphasic contraction pulses and the inversion of contraction pulses were seen when calcium was removed from the gut of the animal. It was concluded that the contraction pulse was a calcium spike. The action of urethan thus may be on the calcium mechanism. However, it is more reasonable to conclude that urethan causes a transient transport defect, or damage to the tissue in much the same way that absence of calcium caused damage. This conclusion is further supported by unpublished experiments with animals which were starved for three weeks and by experiments with animals which were exposed to nitrogen mustard. In the experiments with nitrogen mustard and in the experiments with long-term starvation, the same result was obtained; that is, contraction pulses consistently became very small, typically became biphasic and frequently were seen to be positive going rather than the usual negative going spikes. Consequently, the effect on contraction pulses observed with urethan, calcium, and nitrogen mustard, and those due to starvation are probably all the same effect. It is probably a membrane damage effect and not a specific ion effect. The removal of calcium typically increases membrane permeability and we therefore conclude that these results are due to a generalized increase in membrane permeability leading to a diminution of contraction pulses and their biphasic nature. Also, since the contraction pulse is considered to be generated on the inward facing cell layer, it is immune to external medium ionic concentrations unless the solute is freely permeable. This is true for urethan, probably true for nitrogen mustard over a long-term period, and is consistent with the finding that calcium had to be removed from the gut before the effect was seen, whereas short-term calcium re-

moval from the external medium did not produce this effect. The effect on CP's in all experimental manipulations is reversible.

The effect of urethan on action potentials in nervous tissue which has been reported extensively (Barker and Gainer, 1973; Il'inskii and Tertyshnik, 1960; Iwase, 1957; Kobayashi, 1963; Rudolph, 1963; Salmoiraghi and Weight, 1967; Sorokina, 1958; Thesleff, 1956) is only partly consistent with the results with hydra. That is, whereas action potentials disappeared when urethan in concentrations of 1 to 3% is used, membrane potentials stabilize in the other preparations. However, transepithelial potentials in hydra do not stabilize but change significantly.

Urethan has a more extensive effect on hydra than the relaxation of myotomes, which is the typical casual observation of most investigators. The experiments summarized in Table II clearly show that urethan causes the inner muscle cell layer in the gastrodermal layer to actively contract. This was demonstrated two ways: when the regenerate is intact, it lengthens in the presence of urethan over its maximal relaxed length without urethan; when a longitudinal cut is made, urethan is still able to cause an increase in length of the cut segment. However, the cut segment cannot achieve the length of the uncut segment (Experiment 1, Table II). This strongly suggests that for the animal to fully relax, the internal muscle layer must be intact. Therefore, lengthening or relaxation for hydra is probably a two-step process involving both contraction of the inner muscle layer and relaxation of the outer longitudinal muscle layer. The reason that corresponding electrical activity is not recorded for the elongation of the animal has never been clear, although it is apparent that the inner muscle cell layer is, in fact, involved in animal elongation.

In summary, urethan not only has a profound effect on the transepithelial potential and therefore the electrogenic sodium transport mechanism of hydra, but it also has an effect on the gastrodermal myotomes causing active contraction of that muscle layer.

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Summary

1. The relaxed length of hydra is increased in the presence of urethan. This suggests a direct effect of urethan on the inner circular myotome layer.

2. Urethan causes the normal transepithelial resting potential to reverse such that the gut becomes negative relative to the external medium.

3. Urethan also causes contraction pulses to become reduced in magnitude.

4. It is suggested that urethan has both a direct effect on the hydra myotomes and on the sodium transport mechanism.

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